

BIANCA MARIA UVA*, MADDALENA STURLA* &
MARIA ANGELA MASINI*

KIDNEY AND OSMOREGULATION IN
SPELEOMANTES GENEI (TEMMINCK & SCHLEGEL, 1838)
(AMPHIBIA, PLETHODONTIDAE)

INTRODUCTION

Vertebrates that live in hypoosmotic environments maintain the equilibrium between extracellular and intracellular compartments excreting the excess of water and reabsorbing ions. Amphibians are the first Vertebrates that conquered with some success the dry land; some spend much of their life on the ground but return to water periodically to reproduce. Some salamanders have adopted a variety of adaptations to avoid the necessity of returning to water, but very few succeeded. Plethodontid salamanders like *Speleomantes genei* (Temminck & Schlegel, 1838), are among the few that were successful in laying their eggs on the ground of humid caves. They live in dark limestone caves where the temperature is fairly stable (LANZA 1946), they are lungless and possess an excellent olfactory apparatus that allows to catch preys in the darkness (UVA & DEPLANO 1983; UVA 1985). Ions reabsorption and water handling must therefore be a fundamental priority.

Aim of the present research was to investigate on the presence and localisation of ion transport and water channel proteins comparing in the kidneys tubules of *Speleomantes genei* the histological architecture with the immunohistochemical results.

Sodium ions, brought from the lumen into the cells via $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransport proteins, are transported by the sodium pump in the extracellular compartment. Water may follow passively. Recently

* Dipartimento di Biologia, Università di Genova, Viale Benedetto XV n. 5, 16132 Genova, Italia.

a family of water channel proteins, the aquaporins, that rules the transmembrane trafficking of water, was identified (AGRE *et alii* 1993). Calcium ions are also reabsorbed in the kidney tubules. The mitochondrial density was correlated to the presence of the osmoregulatory proteins in the cells of the tubules.

The investigation was carried out by histological techniques and immunohistochemistry using antibodies to $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransport proteins, Na^+/K^+ ATPase and Ca^{2+} ATPase, the water channel protein AQP₃ and the antibody to the inner mitochondrial membrane (AMA).

MATERIALS AND METHODS

Animals and tissue preparation

Six specimens of *Speleomantes genei* were collected in Sardinian caves and housed in a tank with mud and sand. Within a few days the animals were killed by a blow to the head and spinal cord transection. The kidneys were immediately removed at 4°C, fixed in Bouin's fluid and then processed for embedding in paraffin wax.

Animal manipulation was performed according to the recommendations of the Ethical Committee and under the supervision of authorised investigators.

Histology

Bouin fixed sections (5µm thick) were stained with the haematoxylin-eosin technique.

Immunohistochemistry

Sections (5µm thick) from Bouin-fixed samples were subjected to the indirect immunofluorescence technique (COONS *et alii* 1955). Sections were dewaxed and after exposure in a moist chamber to Normal Goat Serum (diluted 1:50; Dako, Santa Barbara, CA) at 20°C, the unwashed sections were incubated overnight at 4°C with the following antisera: Ab-AMA (Antibody to Mitochondrial Antigen) raised in rabbit (diluted 1:20; Medic, Pavone Canavese, Italy), anti-water channel aquaporin 3 (AQP3) raised in rabbit (diluted 1:300;

Sigma-Aldrich, Milano, Italy), Ab- Na^+K^+ -ATPase, subunit, Ab to Ca^{2+} ATPase, and Ab- $\text{Na}^+/\text{K}^+/\text{Cl}^-$ (T_4 NKCC1) cotransporter raised in mouse (these antibodies were obtained from the Developmental Studies Hybridoma Bank-Department of Biological Sciences, University of Iowa, Iowa City, IA). After washing in phosphate buffered saline (PBS, 0.1 M, pH 7.4), a second layer of fluoresceine-isothiocyanate conjugated -globulins, swine anti-rabbit (diluted 1:20, Dako) for the Ab-AMA, Ab-AQP3, and goat anti-mouse (diluted 1:100, Sigma-Aldrich) for the Ab- Na^+K^+ -ATPase, Ca^{2+} ATPase and $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporters treated sections, was applied for 30 min in a moist chamber, at 20°C. Sections were rinsed in PBS, mounted with glycerol-PBS (1:9) and examined under an Olympus epifluorescence microscope. The specificity of the immunostaining was verified by omitting one of the steps of the immunohistochemical procedure, or by replacing the primary antiserum with non-immune serum or PBS.

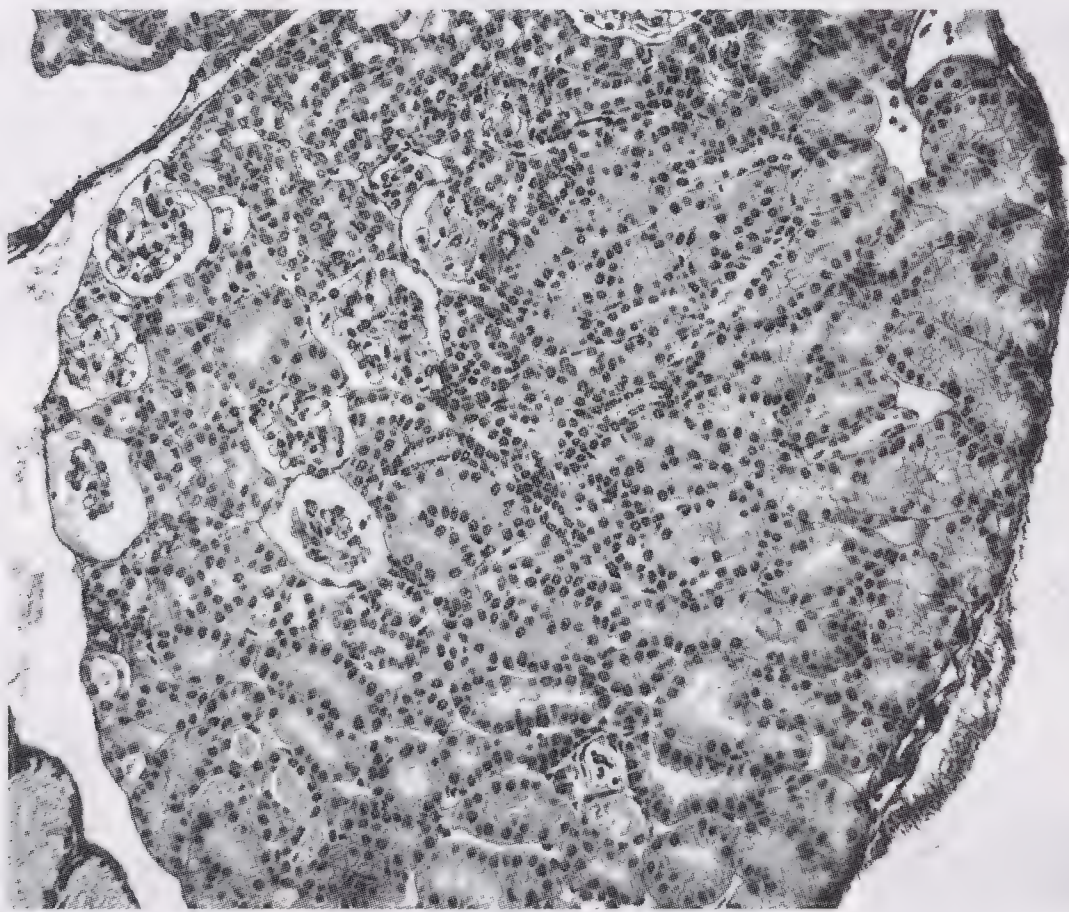


Fig. 1 - Photomicrograph of a transverse section of *Speleomantes genei* kidney to show the general arrangement of the tissue. Haematoxylin-eosin x 40.

RESULTS

Histology

The renal tubule consists of a big corpuscle, a long proximal segment, a distal segment and a collecting duct; two narrow ciliated

segments connect the glomerular capsule with the proximal segment and the latter with the distal segment (fig. 1 and fig. 2). The long proximal segment consists of high cells with basal nucleus, a brush border and vesicles in the apical region, protruding toward the lumen, in which large quantities of materials are secreted (fig. 2 A). The distal segment consists of moderately high cells with no brush border in the apical region (fig. 2 B). The collecting duct consists of low cuboidal cells, deprived of brush border and with very few membrane infoldings at the basal side (fig. 2 C).

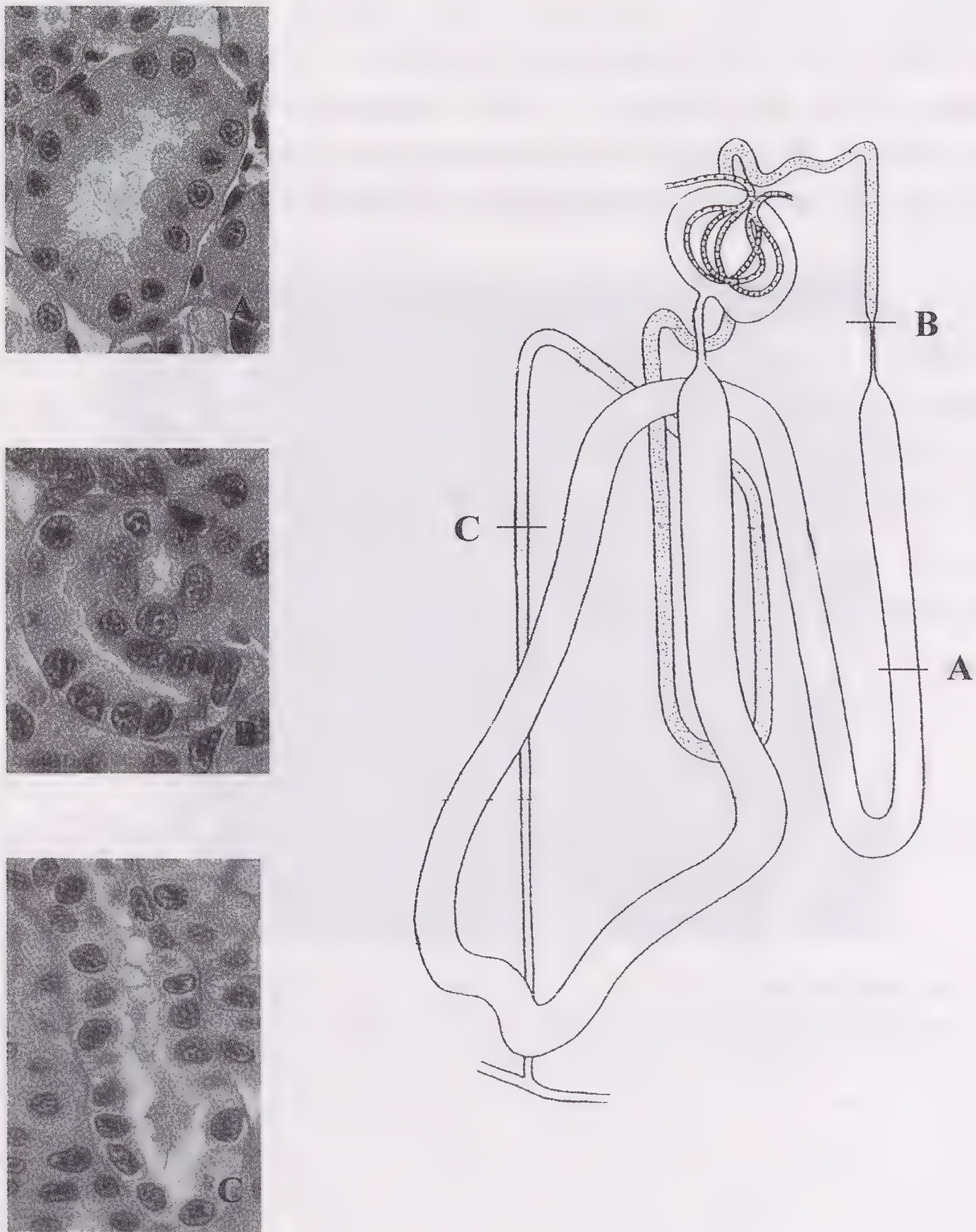


Fig. 2 - Diagrammatic reconstruction of a renal tubule. (A) section of the proximal region showing apical vesicles and material into the lumen. (B) section of the distal segment with basal nucleus and clear apical cytoplasm. (C) section of the collecting duct with low cuboidal cells. A,B,C x 100.

Immunohistochemistry

$\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporter. Immunoreactivity for the cotransport proteins was distributed in the cytoplasm of the cells in the proximal and distal segments. In the collecting duct the positivity was mainly localised at the apical region of the cells (fig. 3 a).

Na^+K^+ -ATPase. Immunoreactivity for the sodium pump was limited to the distal segments, at the basal-lateral side of the cells (fig. 3 b).

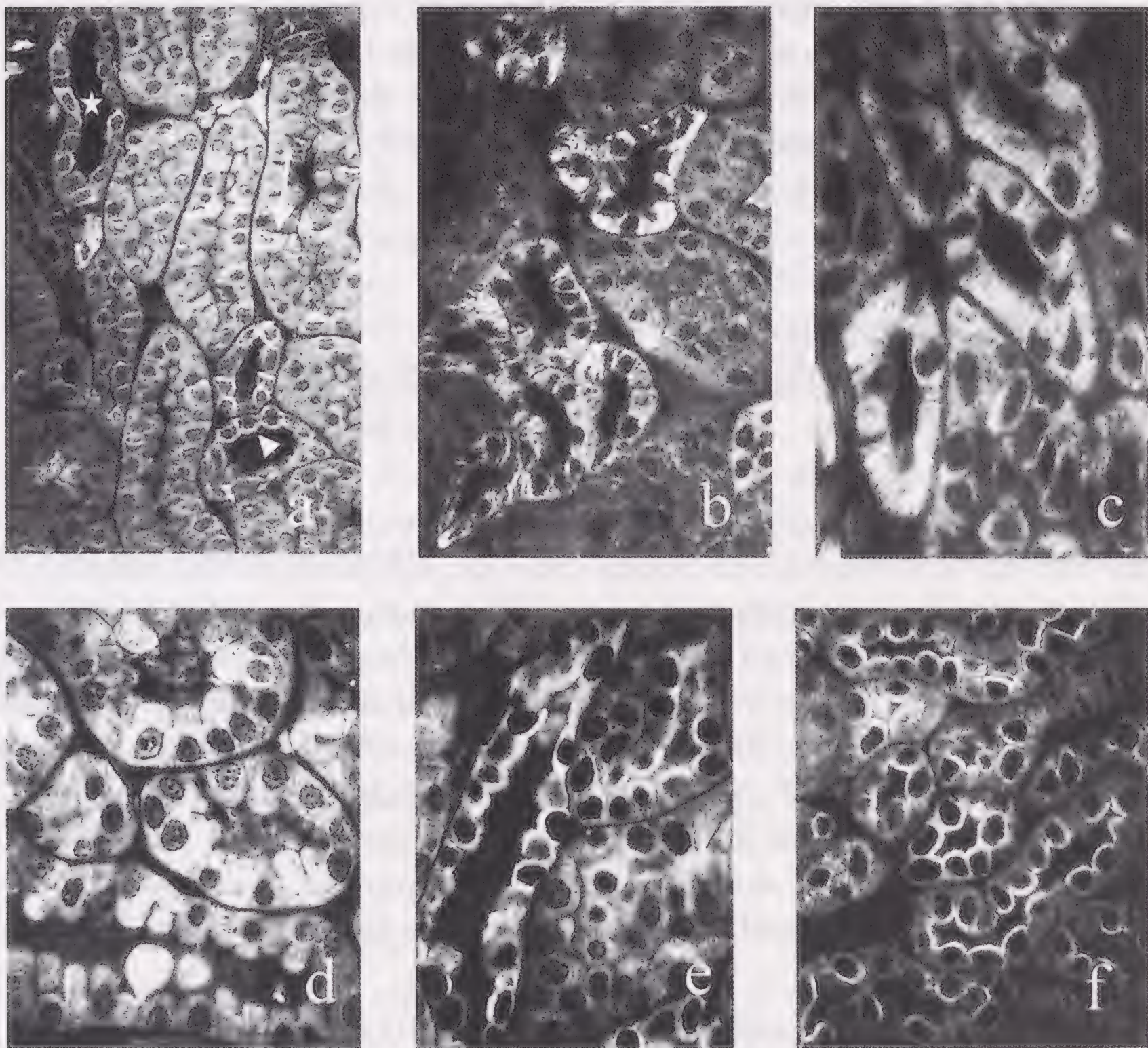


Fig. 3 - (a) Immunoreactivity for the cotransport proteins in the proximal and distal segments (arrow and arrowhead respectively) and in the collecting ducts (star). (b) Immunoreactivity for Na^+K^+ -ATPase in the distal segments located at the baso-lateral sides of the cells. (c) Immunoreactivity for Ca^{2+} -ATPase distributed in all the cytoplasm of the cells in the distal segments. (d) Immunoreactivity for AQP_3 in the cells of the proximal segment and (e) in the cells of the collecting ducts. (f) Immunostaining for AMA located at the luminal side of the cells in the collecting ducts. a x50; b x70; c, d, e, f x100.

Ca²⁺ATPase. The immunostaining was localised in the cytoplasm of the distal segment's cells (fig. 3 c)

Aquaporins. The reaction was distributed throughout the cytoplasm in the cells of the proximal tubules and limited to the luminal side of the cells in the collecting ducts, where the immunoreactivity was very strong. In the proximal tubule AQP3 is also located in intracellular vesicles protruding into the lumen of the plasma membrane (figs. 3 d, e).

Mitochondria. Immunostaining for AMA (proteins of the inner mitochondrial membrane) was localised in the cells of the proximal tubules, gathered around the nucleus, in the distal segment, located in the basal part of the cells, and in the collecting ducts where mitochondria were concentrated at the luminal side (fig. 3 f).

DISCUSSION AND CONCLUSIONS

The architecture of the kidney in diverse species of Amphibians shows no difference: the nephrons consists of the same segments and the shape of the cells, in each segment, are very similar. In consequence, the maintenance of the ion-water equilibrium in the diverse environmental conditions must be allocated in the osmoregulatory mechanisms, present in the different regions of the nephron. Na⁺/K⁺ATPase and Na⁺/K⁺/Cl⁻ cotransporters are the major proteins involved in ion transport across the kidney tubules. Na⁺/K⁺ATPase is, in most eukaryotic cells, the enzymatic activity responsible for the active transport of Na⁺. The enzyme has been shown to consist of two subunits α and β . The α subunit is the catalytic part of the Na⁺/K⁺ exchange and shows a highly conserved aminoacid sequence in diverse vertebrate and invertebrate species (KAWAKAMI *et al.* 1985; SHULL *et alii* 1985).

The family of Aquaporins (AQPs) consists of ten proteins cloned from lower organisms, like yeast, to mammals (for a review see AGRE 2000; VERKMAN & MITRA 2000). Two aquaporins subgroups appear to be present: AQPs that are selectively permeable to water (AQPs 1,2,4,5,6,8) and aquaglyceroporins (AQPs 3,7) that are permeable both to water and glycerol (VERKMAN & MITRA 2000). Functional expression of aquaporin 3 in *Xenopus* oocytes confirmed its predominant water-channel function in Amphibians. AQP3 mRNA was

mainly expressed in rat kidney in the cells of the collecting duct (ISHIBASHI *et alii* 1994).

In *Speleomantes*, immunoreactivity for the cotransporter NKCC1 T₄, was limited to the distal segment and the collecting duct, segments involved in ion and water reabsorption. The monoclonal antibody to the Na⁺/K⁺ATPase, IGg α5, recognised its epitope in the cells of the distal segment. Immunoreactivity for AQP3 was very strong and distributed in the proximal and distal segments. The presence in the proximal segments' cells of AQP3-filled vesicles protruding into the lumen may indicate a fusion by exocytosis with the plasma membrane, thus inserting AQP3 into the membrane. This process was described for AQP2 in mammalian renal cells (BROWN *et alii* 1998).

It may be concluded that *Speleomantes*, that spends its life on land, reabsorbs from the preurine, by means of the collecting ducts cells, water and, moderately, ions from the proximal and distal segments. A similar function is seen in the segments of the kidney of some Birds and in Mammals.

ACKNOWLEDGEMENTS

This work was supported by the University of Genoa, Italy. The antibody developed by Fambrough D.M. was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

REFERENCES

- AGRE P., 2000 - Aquaporin water channels in kidney - *J. Am. Soc. Nephrol.*, Philadelphia, 11: 764-777.
- AGRE P., PRESTON G.M., SMITH B.I., JUNG J.S., RAINA S., MOON C., GUGGINO W.B. & NIELSEN S., 1993 - Aquaporin CHIP: the archetypal molecular water channel - *Am. J. Physiol. Renal*, Bethesda, 265: F463-F476.
- BROWN D., KATSURA T. & GUSTAVSON C.E., 1998 - Cellular mechanisms of aquaporin trafficking - *Am. J. Physiol. Renal*, Bethesda, 275: F328-F331.
- COONS A.H., LEDUC E.H. & CONNOLLY J.M., 1955 - Studies on antibody. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit - *J. exper. Med.*, New York, 102: 49-59.

- ISHIBASHI K., SASAKI S., FUSHIMI K., UCHIDA S., KUWAHARA M., SAITO H., FURUKAWA I., NAJIMA K., YAMAGUCHI Y., GOJOBORI T. & MARUMO F., 1994 - Molecular cloning and expression of a member of the aquaporin family with permeability to glycerol and urea in addition to water expressed at the basolateral membrane of kidney collecting duct cells - *Proc. natl. Acad. Sci. U.S.A.*, Washington, 91: 6269-6273.
- KAWAKAMI K.S., NOGUCHI M., TAKAHASCHI H., OHTA T., KAWAMURA M., NOJIMA H., NAGANO K., HIROSE T., INAYAMA S., HAYASHIDA H., MIYATA T. & NUMA S., 1985 - Primary structure of the α -subunits of *Torpedo californica* (Na^+/K^+): ATPase deduced from cDNA sequence - *Nature*, London, 316: 733-736.
- LANZA B., 1946 - L'*Hydromantes* Gistel in Toscana e notizie sui suoi costumi - *Arch. zool. ital.*, Torino, 31: 219-237.
- SHULL G.E., SCHWAT A. & LLINGREL J.B., 1985 - Amino-acid sequence of the catalytic subunit of the Na^+/K^+ -ATPase deduced from a complementary DNA - *Nature*, London, 316: 691-695.
- UVA B.M., 1985 - Olfaction, related structures and functional adaptations - *Boll. Zool.*, Modena, 52: 331-337.
- UVA B.M. & DEPLANO S., 1983 - Olfactory structures in *Hydromantes italicus*: a morphological basis for a particular prey catching behaviour - *Boll. Zool.*, Modena, 50: 57-62.
- VERKMAN A.S. & MITRA A.K., 2000 - Structure and function of aquaporin water channels - *Am. J. Physiol. Renal*, Bethesda, 278: F13-F28.

ABSTRACT

Speleomantes genei spends its life in humid habitats and completely avoids water even during reproduction, its control of ion-water balance must therefore be a fundamental priority. In Amphibians the kidney is one of the organs involved in the loss of water, being non-mammalian vertebrates unable to produce hyperosmotic urine. To reduce water and ion loss the cells in the renal tubules must possess well-developed osmoregulatory machinery.

Sodium ions, brought into the cells via $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransport proteins, are extruded by the sodium pump Na^+/K^+ ATPase in the extracellular compartments. The water channel proteins, aquaporins, rule the transmembrane trafficking of water. Ca^{2+} ATPase control the reabsorption of calcium ions. The presence of ion cotransport proteins, sodium pump, Ca^{2+} ATPase and aquaporins were investigated in the different regions of the renal tubule. The mitochondrial density was correlated to the presence of the osmoregulatory proteins.

The investigation was carried out by histological staining to identify the segments of the nephron and by immunohistochemistry using antibodies to $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransport proteins NKCC1 T_4 , to Na^+/K^+ ATPase, to Ca^{2+} ATPase, to the water channel aquaporin 3 and to proteins of the inner mitochondrial membrane AMA.

The results indicate that ion reabsorption occurs in the distal and collecting ducts, while water channels are located in the proximal segment and in the collecting duct of the nephron.

In *Speleomantes* therefore different segments of the renal tubule are specialised to perform diverse functions, all of which are cooperating to the maintenance of ion-water balance.

RIASSUNTO

Rene ed osmoregolazione in *Speleomantes genei* (Temminck & Schlegel, 1838) (Amphibia, Plethodontidae).

Speleomantes genei trascorre la sua vita in ambienti umidi completamente svincolato dall'ambiente acquatico anche durante la riproduzione. La difesa del patrimonio idrico-salino deve quindi necessariamente essere un problema prioritario. Il rene è tra gli organi più coinvolti nella perdita d'acqua poiché i Vertebrati non mammiferi producono un'urina ipoosmotica rispetto al plasma. Per ridurre la perdita d'acqua e di ioni le cellule del tubulo renale devono possedere un ben sviluppato sistema osmoregolativo. L'ultrafiltrazione glomerulare lascia passare gli ioni nella preurina, proteine cotrasportatrici, quali la $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporter, trasferiscono gli ioni sodio dal lume all'interno delle cellule del tubulo renale. La pompa sodica $\text{Na}^+/\text{K}^+\text{ATPasi}$ estrude gli ioni sodio nel comparto extracellulare. Le aquaporine, canali per l'acqua, regolano il trasporto delle molecole d'acqua attraverso le membrane plasmatiche. La $\text{Ca}^{2+}\text{ATPasi}$ controlla il riassorbimento degli ioni calcio.

In questa ricerca la presenza delle proteine cotrasportatrici, della pompa sodica, della $\text{Ca}^{2+}\text{ATPasi}$, e dell'aquaporina è stata studiata nelle differenti regioni del tubulo renale. La densità mitocondriale è stata correlata alla presenza delle proteine coinvolte nell'osmoregolazione. La ricerca è stata condotta con colorazioni istologiche per identificare i differenti segmenti del tubulo renale e con metodiche d'immunistoichimica utilizzando anticorpi contro la proteina cotrasportatrice $\text{Na}^+/\text{K}^+/\text{Cl}^-$ NKCC1 T4, gli enzimi $\text{Na}^+/\text{K}^+\text{ATPasi}$ e $\text{Ca}^{2+}\text{ATPasi}$, la proteina aquaporina 3 ed anticorpi contro proteine della membrana mitocondriale interna AMA. I risultati indicano che il riassorbimento attivo degli ioni avviene nel segmento distale e nel dotto collettore, mentre i canali per l'acqua sono localizzati nel segmento prossimale e nel dotto collettore. In *Speleomantes* quindi i diversi segmenti del tubulo renale sono specializzati per eseguire funzioni differenti, tutte però coordinate al mantenimento dell'equilibrio idrico-salino.